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Abstract: Recognition of pathogen-associated molecular patterns that activate IL-1 is regulated by inflammasomes, predominantly of the nucleotide-binding oligomerization domain-like receptor (NLR) family. NLRP3 inflammasome is involved in the innate immune responses in periodontal disease. This is an inflammatory condition that destroys the tooth-supporting (periodontal) tissues, initiated by the subgingival formation of multi-species biofilms, frequently including the Gram-negative species Porphyromonas gingivalis. The aim of this study was to investigate the relative effect of P. gingivalis as part of subgingival biofilm, on the expressions of NLRP3 inflammasome, absent in melanoma (AIM)2 (a non-NLR inflammsome) and IL-1 by human gingival fibroblasts. The 10-species subgingival biofilm model, or its 9-species variant excluding P. gingivalis, were used to challenge the cells for 6 h. Gene expression analysis for various inflammasome components and IL-1 was performed by TaqMan real-time PCR. The 10-species subgingival biofilm reduced NLRP3 and IL-1 , but did not affect AIM2 expression. Exclusion of P. gingivalis from the biofilm partially rescued NLRP3 and IL-1 expressions. In conclusion, subgingival biofilms down-regulate NLRP3 and IL-1 expression, partly because of P. gingivalis. These dampened host innate immune responses may favour the survival and persistence of the associated biofilm species in the periodontal tissues.

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Down-regulation of NLRP3 inflammasome in gingival fibroblasts by subgingival biofilms: involvement of *Porphyromonas gingivalis*

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ABSTRACT

Recognition of pathogen-associated molecular patterns that activate interleukin (IL)-1β is regulated by inflammasomes, predominantly of the nucleotide-binding oligomerization domain-like receptor (NLR) family. NLRP3 inflammasome is involved in the innate immune responses in periodontal disease. This is an inflammatory condition that destroys the tooth-supporting (periodontal) tissues, initiated by the subgingival formation of multi-species biofilms, frequently including the Gram negative species *Porphyromonas gingivalis*. The aim of this study was to investigate the relative effect of *P. gingivalis* as part of subgingival biofilm, on the expressions of NLRP3 inflammasome, absent in melanoma (AIM)2 (a non-NLR inflammsome), and IL-1β by human gingival fibroblasts. The 10-species subgingival biofilm model, or its 9-species variant excluding *P. gingivalis*, were used to challenge the cells for 6 h. Gene expression analysis for various inflammasome components and IL-1β was performed by TaqMan real-time polymerase chain reaction. The 10-species subgingival biofilm reduced NLRP3 and IL-1β, but did not affect AIM2 expression. Exclusion of *P. gingivalis* from the biofilm partially rescued NLRP3 and IL-1β expressions. In conclusion, subgingival biofilms down-regulate NLRP3 and IL-1β expression, partly attributed to *P. gingivalis*. These dampened host innate immune responses may favour of survival and persistence of the associated biofilm species in the periodontal tissues.

INTRODUCTION

Periodontal diseases are characterised by the inflammatory destruction of the tooth supporting (periodontal) tissues, and they are the most common chronic inflammatory diseases in man. This cluster of diseases is attributed to resident oral bacteria colonizing the tooth surfaces in the form of polymicrobial biofilm communities [1]. Interaction of biofilms with the juxtaposing periodontal tissues triggers an inflammatory response, which aims to prevent bacterial colonization and establishment [2]. However, if the inflammatory response becomes excessive, it will damage the periodontal tissues, rather than being protective [3]. Periodontitis is a form of periodontal disease in which the inflammatory response has progressed enough to destroy the tooth-supporting alveolar bone, eventually leading to tooth loss, if left untreated. The causative factor of periodontitis is the development of a "subgingival" biofilm, in other terms of a biofilm that forms below the gingival margin within a periodontal pocket, consisting of characteristic bacterial species [4-6]. *Porphyromonas gingivalis* is a black-pigmenting Gram-negative anaerobe very frequently detected in subgingival biofilms from sites with periodontitis [7]. Interestingly, even at low colonization levels, *P. gingivalis* has a key role in altering the composition of the local oral commensal microbiota, which is also required for *P. gingivalis*-induced bone loss [8]. This species is considered notorious for its capacity to manipulate host cell signalling, either by promoting or by dampening inflammatory innate immune responses [9]. Hence *P. gingivalis* can orchestrate a deregulation of the physiological host-microbial homeostasis [10].

Interleukin (IL)-1 cytokines are key modulators of the inflammatory responses in periodontal diseases [11]. Clinical studies indicate that IL-1β, the best known member of this family, is detected at higher levels in gingival crevicular fluid [12] or gingival tissues [13] of patients with periodontal disease, compared to healthy subjects. On the cellular level, activation and production of IL-1β is controlled by the "inflammasomes", which are intracellular protein complexes that can sense pathogen-associated molecular patterns (PAMPs) [14]. The nucleotide-binding oligomerization domain-like receptor (NLR) inflammasomes are intracellular pattern recognition receptors (PRRs) that detect PAMPs. Through the recruitment of cysteine proteinase caspase-1, NLRs activate intracellularly stored pro-IL-1β, which is subsequently released from the cell. The NLRP3 inflammasome in particular, consists of three components: the NLRP3 "sensor", the caspase-1 "effector", and the apoptotic speck

protein containing a C-terminal caspase recruitment domain (ASC) "adaptor" that links former two molecules. NLRP3 is activated by cell stresses [15] and bacteria or virus [16-19]. Recent clinical evidence demonstrates that the expression of this inflammasome is higher in periodontal diseases, compared to healthy ones [13]. Absent in melanoma (AIM)2 is a non-NLR inflammasome that senses double-stranded DNA from various sources, including bacteria, viruses or host cells [20]. A shared feature of the NLRP3 and AIM2 inflammasomes is that they both include ASC and caspase-1 [21].

Gingival fibroblasts (GF) are the major population of the gingival tissue, which is one of the constituents of periodontal tissues. They are responsible for the synthesis and degradation of the extracellular matrix, and respond to PAMPs by producing mediators of inflammation [22]. In doing so, they are crucial for regulating the homeostasis of the periodontal tissues in health and disease. It was recently shown that GF express NLRP3 and AIM2, which are differentially regulated by *in vitro* supragingival and subgingival biofilms supernatants. Subgingival biofilm supernatants caused a down-regulation of NLRP3 expression, but had a bi-phasic effect on AIM2 and IL-1β expression [23]. This "dampened" host sensing elicited by subgingival biofilms is perceived as a strategy for evading immune surveillance, which may promote pathogen survival. Although it is postulated that *P. gingivalis*, as part of polymicrobial subgingival biofilms, is involved in this effect, this has not yet been demonstrated. Therefore, the present *in vitro* study aims to investigate the involvement of *P. gingivalis* in the regulation of NLRP3 and AIM2 inflammasomes in GF, by subgingival biofilms.

MATERIALS AND METHODS

In vitro *biofilm model*

The 10-species *in vitro* "subgingival" Zürich biofilm model [23, 24] used in this study, consisting of *Campylobacter rectus* (OMZ 697), *Fusobacterium nucleatum* (OMZ 596), *Porphyromonas gingivalis* ATCC 33277^T (OMZ 925), *Prevotella intermedia* ATCC 25611^T (OMZ 278), *Tannerella forsythia* OMZ 1047, *Treponema denticola* ATCC 35405^T (OMZ 661), *Veillonella dispar* ATCC 17748^T (OMZ 493), *Actinomyces oris* (OMZ 745), *Streptococcus anginosus* (OMZ 817), and *Streptococcus oralis* SK 248 (OMZ 607). A 9-species version of this biofilm was also grown, in the absence of strain *P. gingivalis* ATCC 33277^T (OMZ 925). Briefly, the

biofilms were grown in 24-well cell culture plates on sintered hydroxyapatite discs, resembling a natural tooth surface. To achieve pellicle formation, these surfaces were pre-conditioned for 4 h with 800 µl foetal bovine serum (FBS) diluted 1:1 in sterile 25 % NaCl. To initiate biofilm formation, the hydroxyapatite discs were covered for 16.5 h with 1.6 ml of growth medium consisting of 70 % FBS (diluted 1:10) and 30 % FUM medium [25] containing 0.3 % glucose, and 200 µl of a bacterial cell suspension containing equal volumes and densities from each strain. After 16.5 h of anaerobic incubation at 37 °C, the inoculum suspension was removed from the discs by "dip-washing" using forceps, transferred into wells with fresh medium (70 % FBS diluted 1:10, and 30 % FUM containing 0.15 % glucose - 0.15 % sucrose), and incubated for further 48 h in anaerobic atmosphere. During this time-period, the discs were "dip-washed" three times and the biofilm cultures growing on them were given fresh medium once daily. After a total 64.5 h of incubation, one biofilm-carrying hydroxyapatite disc was placed carefully in each cell culture well, with the biofilm-coated surface facing towards the GF cell monolayer. Analysis of bacterial composition of the biofilms at this time-point is provided in Table 1, and was performed by bacterial cell culture, or immunofluorescence (IF), or fluorescent *in situ* hybridization (FISH), as previously described [24, 26, 27]. A plastic ring support ensured a distance of 1 mm between the biofilm-carrying hydroxyapatite disc and the underlying GF cell monolayers, allowing fluid flow. As controls, pellicle pre-coated hydroxyapatite discs were used that did not contain biofilm cultures. Upon completion of the experiments, after 6 h of challenge, the hydroxyapatite discs were removed from the cultured and the GF cell monolayers were processed for the subsequent analyses, as described below.

Cell cultures

Primary human GF cell lines were established as previously described [28]. Briefly, gingival tissue biopsies used were obtained from a healthy young individual, who had their first premolar removed during the course of orthodontic treatment. Ethics approval was granted by the Human Studies Ethical Committee of Umeå University, Sweden, and informed consent was given by the donor. The cells were passaged and cultured in Minimum Essential Medium Alpha (Gibco), supplemented with 5 % heat-inactivated foetal bovine serum (Sigma), 50 U/ml penicillin, and 50 µg/ml streptomycin (Sigma). For the experiments, GF at passage 4 were seeded at

concentration 1 x 10^4 cells/cm^2 in antibiotics-free culture medium, supplemented with and 5 % FBS. The cells were allowed to attach overnight, maintaining a sub-confluent status, and then cultured for 6 h in the presence or absence of either biofilm.

RNA extraction and cDNA synthesis

Upon completion of the experiments, after 6 h of challenge, the culture supernatants were removed and the cell monolayers were washed twice in PBS, before being lysed. Total RNA was extracted by the RNeasy Mini Kit (QIAGEN), and its concentration was measured by a NanoDrop spectrophotometer. One μg of total RNA was then reverse transcribed into single-stranded cDNA by M-MLV Reverse Transcriptase, $Oligo(dT)_{15}$ Primers, and PCR Nucleotide Mix (Promega), at 40°C for 60 min, and 70°C for 15 min. The resulting cDNA was stored at -20°C.

Quantitative real-time Polymerase Chain Reaction (qPCR)

Gene expression analysis was performed by qPCR, in an ABI Prism 7000 Sequence Detection System and software (Applied Biosystems). For the amplification reactions, TaqMan Gene Expression Master Mix and Gene Expression Assay kits (Applied Biosystems) were used (assay IDs: NLRP3: Hs00918085-m1, ASC: Hs01547324-m1, caspase-1: Hs00354836-m1, AIM2: Hs00915710-m1, IL-1β: Hs00174097-m1, GAPDH: Hs99999905-m1). The standard PCR conditions were 10 min at 95 ºC, followed 40 cycles at 95 ºC for 15 seconds and 60 ºC for 1 min. GAPDH was used as a housekeeping gene. The expression levels of the target transcripts in each sample were calculated by the comparative Ct method ($2^{-\Delta Ct}$ formula), after normalization to GAPDH.

Measurement of IL-1β by enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1β secreted by GF into the culture supernatant were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (DY201, DuoSet, R&D Systems, Abingdon, UK). Absorbance was measured at 450 nm using a microplate reader (Epoch, BioTek, Luzern, Switzerland). A wavelength correction of 570 nm was used for the subtraction of the background. A

standard curve was created using known concentrations of rhIL-1β provided in the kit. The concentration of IL-1β in each sample was calculated by a four-parameter logistic (4-PL) equation. The lowest detection limit of the assay was 1.7 pg/ml. The results represent the mean ± SEM from four independent cell cultures in each group.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyze the statistical significances of the data. Bonferroni post-hoc test was employed for the comparisons between individual groups. The data were considered significant at $P<0.05$.

RESULTS

The GF cultures were challenged for 6 h with either the 10-species subgingival biofilm, or its 9-species variant, which excluded *P. gingivalis*. The effect of these two biofilms on the gene expression of the NLRP3 and AIM2 inflammasomes, as well as IL-1β was then investigated. The 10-species biofilm caused a significant down-regulation of NLRP3 expression by 33 % (Figure 1). However, it did not affect the expressions of adaptor molecule ASC (Figure 2), or the effector molecule caspase-1 (Figure 3). Moreover, the expression of AIM2 was not affected by the biofilm challenge (Figure 4). Nevertheless, IL-1β expression exhibited a significant down-regulation by 35 % (Figure 5).

The effects elicited by the 10-species biofilm, were compared to those caused by the 9-species biofilm, where *P. gingivalis* was excluded. The bacterial composition of the two biofilms was otherwise comparable, as there were no log-scale differences (Table 1). With regards to the expression of the various inflammasome components, exclusion of *P. gingivalis* from the biofilm partially rescued NLRP3 expression to 85 % of control levels (Figure 1), whereas it did not affect the expression of adaptor ASC (Figure 2), or effector caspase-1 (Figure 3). Accordingly, the expression of AIM2 was not affected (Figure 4). Nevertheless, the expression of IL-1β was partially rescued to 88 % of the control levels (Figure 5), similarly to NLRP3 expression.

The secretion of IL-1β by the cells in response to the 6 h biofilm challenge was also investigated. IL-1β was detected at particularly low levels. In the control

group, IL-1β concentration in the culture supernatant was 5.5 ± 0.4 pg/ml, whereas the presence of the 10-species biofilm reduced this to 4.3 ± 0.3 pg/ml (23 % reduction). In the presence of the 9-species biofilm, which excluded *P. gingivalis*, the IL-1β concentration in the culture supernatant was 3.3 ± 0.1 pg/ml (41 % reduction, compared to control). The concentration of IL-1β in both biofilm groups was significantly lower than the control group. However, the difference between the two biofilm groups did not prove to be statistically significant.

DISCUSSION

This study investigated the effect of *in vitro* subgingival biofilms on inflammasome expression by GF, and evaluated the relative role of *P. gingivalis*. The results indicate a down-regulation of NLRP3 expression by subgingival biofilms, but no effect on the expressions of adaptor ASC and effector caspase-1. These responses are comparable with the effects elicited by subgingival biofilm supernatants, which, at higher concentrations, down-regulated NLRP3, but did not affect ASC or caspase-1 expression [23]. However, lower and mid-range biofilm supernatant concentrations did increase ASC and caspase-1 expressions, rendering this effect as bi-phasic [23]. Overall, the down-regulation of NLRP3, as a PRR sensor, may compromise the capacity of the cells to sense PAMPs, implying a dampening of the endpoint innate immune responses.

The observed effects of the subgibival biofilm on the expression of the NLRP3 inflammasome matches the well established capacity of *P. gingivalis* to manipulate host signalling [9, 29]. This may enable this species to evade host surveillance [30], offering a survival advantage to all co-habiting organisms of the biofilm [9]. For this reason, the present study considered the role of *P. gingivalis* in the observed effects, by establishing in parallel a 9-species biofilm that lacks *P. gingivalis*, and comparing its effects to the 10-species biofilm. The two biofilms did not exhibit quantitative differences in composition, other than the absence of *P. gingivalis*. The data demonstrated that the lack of *P. gingivalis* indeed rescued NLRP3 expression almost to control levels, implying a key role of this species in subgingival biofilms, in down-regulating specific gene expression. Importantly, as a single species, *P. gingivalis* may either up-regulate [13, 31] or down-regulate [32] NLRP3 expression, depending on the cell type. Hence, this indicates that when part of a microbial biofilm community, *P.*

gingivalis may selectively orchestrate the innate immune response, in line with its role as a "keystone" species [8].

The relative effect of *P. gingivalis* as member of a biofilm community was also investigated on IL-1β expression by GF. So far there is evidence that co-infection of host cells with *P. gingivalis* and other putative periodontal pathogens, decreases the IL-1-inducing capacity of the latter [33, 34]. It was recently demonstrated that subgingival biofilm supernatants, in which *P. gingivalis* was present, may exert a bi-phasic effect on IL-1β expression, with an increase by lower concentrations and a decrease to control levels by higher ones [23]. In the present experimental system with viable biofilms, IL-1β expression was decreased when the cells were challenged with the 10-species subgingival biofilm, but was partially rescued when *P. gingivalis* was absent (9-species biofilm). Hence, the regulation of IL-1β expression followed a similar pattern to that of NLRP3 expression. Although the expression of these genes are not necessarily interdependent, these results denote that they can be regulated in a parallel manner by subgingival biofilms, which is also in line with the recent observations [23].

Changes in NLRP3 expression would be expected to result in changes of IL-1β secretion by the cells, in various experimental systems. In the present study, it was confirmed that IL-1β secretion was indeed lower in the biofilm-challenged cell cultures, which is in agreement with a lower NLRP3 expression. In the case where *P. gingivalis* was absent from the biofilm, NLRP3 and IL-1β expressions were partially rescued to control levels. Nevertheless, this was not accompanied by a concomitant resumption of IL-1β secreted protein levels. The reason for this is not clear, but in such a complex experimental system several factors may impose limitations in studying IL-1β protein secretion. For instance, GF produce low levels of IL-1β [35, 36] compared to inflammatory cells, and therefore it is difficult to evaluate efficiently differences in production, particularly at the lower bound of the detection spectrum. Moreover, it is established that *in vitro* subgingival biofilms can degrade pro-inflammatory cytokines, including IL-1β [24], an effect which may compromise the endpoint measurements.

The expression of AIM2, the sensor of the homonymous inflammasome that oligomerizes with ASC and caspase-1 [14], was also investigated. AIM2 recognizes different PAMPs compared to NLRP3, including cytosolic double-stranded DNA [20, 21]. The present data do not identify significant changes in AIM2 expression, in the

presence of either biofilm, although subgingival biofilm supernatants were earlier shown to have a bi-phasic effect [23]. Collectively, it is likely that high concentrations of biofilm supernatants elicit equivalent cell responses to viable biofilms, as seen in this study.

In conclusion, down-regulation of NLRP3 inflammasome expression may constitute a strategy to manipulate the local innate immune inflammatory responses [37]. In the case of subgingival biofilms, this could lead to the evasion of host surveillance by the associated species, with the obvious benefit of their survival into the tissue environment [10, 23]. The present study affirms that subgingival biofilms down-regulate NLRP3 and IL-1β gene expression in GF *in vitro*, and further identifies *P. gingivalis* as a key species in these events. This supports the notion that, as part of polymicrobial communities, *P. gingivalis* can disrupt the host-microbial homeostasis [8], in this case by dampening the pathogen-sensing capacity of the cells via inflammasomes.

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TABLES

Table 1. Bacterial composition of the subgingival biofilms

	10-species	9-species
A. oris	8.0 E6 ± 2.1 E6	6.1 E6 ± 8.6 E6
V. dispar	4.8 E7 ± 3.0 E6	7.9 E7 ± 4.4 E6
F. nucleatum	1.4 E8 ± 3.2 E7	2.1 E8 ± 5.1 E7
S. anginosus	5.6 E7 ± 2.0 E7	4.3 E7 ± 3.4 E7
S. oralis	4.4 E7 ± 1.5 E7	2.3 E7 ± 1.3 E7
P. intermedia	3.8 E7 ± 1.3 E7	2.2 E7 ± 1.0 E7
C. rectus (a)	5.2 E6 ± 7.9 E5	9.8 E6 ± 1.3 E6
P. gingivalis	2.0 E7 ± 1.6 E7	---
T. forsythia (b)	2.6 E5 ± 2.3 E5	5.5 E5 ± 8.2 E5
T. denticola (c)	8.0 E2 ± 6.2 E2	4.0 E2 ± 0.0 E2

The presence and numerical composition of the individual bacterial species in the biofilms, just prior to the addition on the cell cultures, was defined by bacterial culture analysis (colony forming unit measurement), or FISH, or IF, as previously described [24, 26, 27]. (a) The detection and counting of *C. rectus* was performed by IF, using monoclonal antibody 212WR2. (b) The detection and counting of *T. forsythia* was performed by IF, using monoclonal antibody 103BF1.1. (c) The detection and counting of *T. denticola* was performed by FISH, using DNA probe TrepG1-679-Cy3 (5' to 3' sequence: GATTCCACCCCTACACTT). The data represents the bacterial mean counts ± SD from triplicate biofilm cultures.

FIGURES AND FIGURE LEGENDS

Figure 1

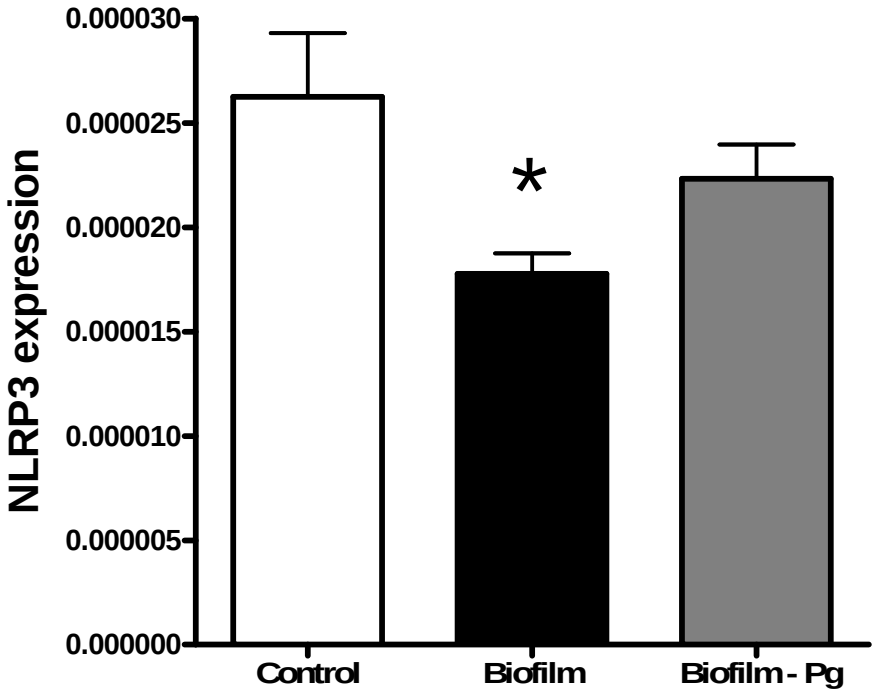

Figure 1. *Regulation of NLRP3 gene expression in response to biofilm challenge.* GF cultures were challenged for 6 h with the 10- or 9-species (excluding *P. gingivalis*) subgingival biofilm. NLRP3 gene expression was calibrated against GAPDH, and presented as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from four independent cell cultures in each group. Asterisk represents statistically significant difference compared to the control group.

Figure 2

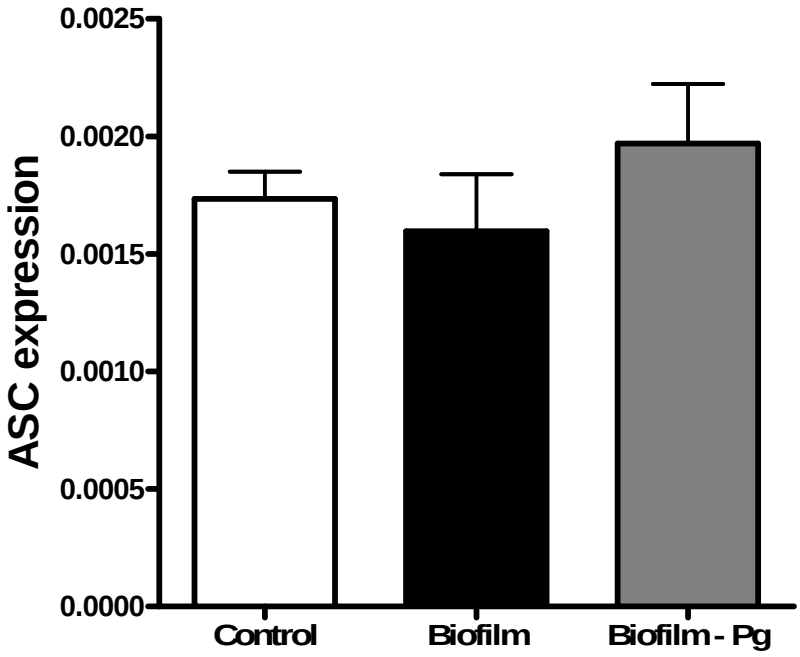

Figure 2. *Regulation of ASC gene expression in response to biofilm challenge.* GF cultures were challenged for 6 h with the 10- or 9-species (excluding *P. gingivalis*) subgingival biofilm. ASC gene expression was calibrated against GAPDH, and presented as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from four independent cell cultures in each group. Asterisk represents statistically significant difference compared to the control group.

Figure 3

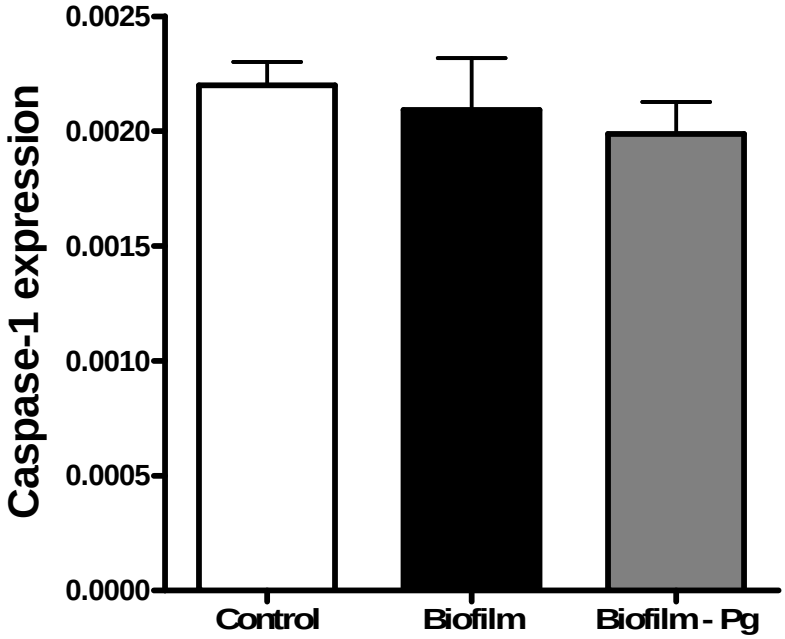

Figure 3. *Regulation of caspase-1 gene expression in response to biofilm challenge.* GF cultures were challenged for 6 h with the 10- or 9-species (excluding *P. gingivalis*) subgingival biofilm. Caspase-1 gene expression was calibrated against GAPDH, and presented as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from four independent cell cultures in each group. Asterisk represents statistically significant difference compared to the control group.

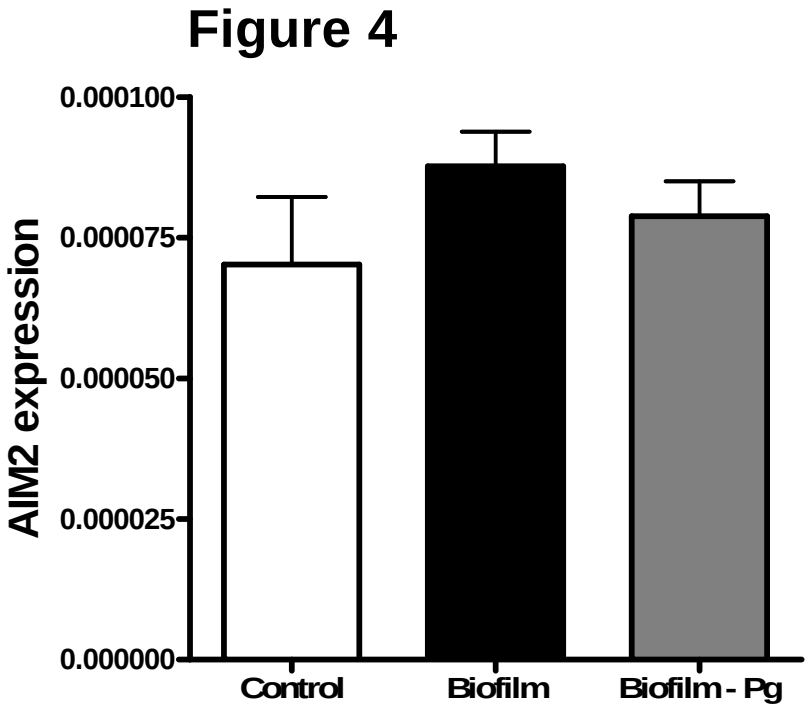

Figure 4. *Regulation of AIM2 gene expression in response to biofilm challenge.* GF cultures were challenged for 6 h with the 10- or 9-species (excluding *P. gingivalis*) subgingival biofilm. AIM2 gene expression was calibrated against GAPDH, and presented as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from four independent cell cultures in each group. Asterisk represents statistically significant difference compared to the control group.

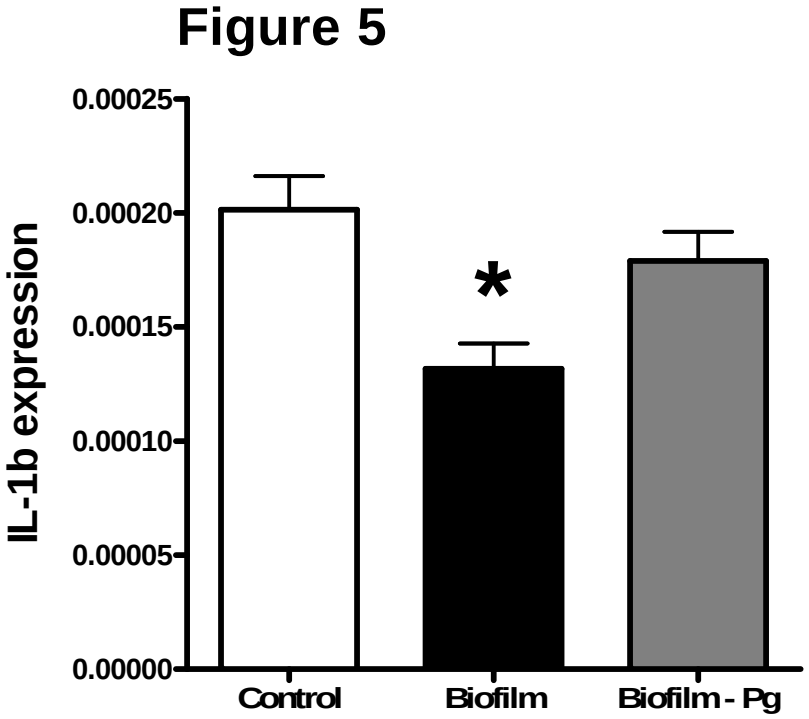

Figure 5. *Regulation of IL-1β expression in response to biofilm challenge.* GF cultures were challenged for 6 h with the 10- or 9-species (excluding *P. gingivalis*) subgingival biofilm. IL-1β gene expression was calibrated against GAPDH, and presented as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from four independent cell cultures in each group. Asterisk represents statistically significant difference compared to the control group.